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Serological Relationships among Members of the Order Carnivora[1]

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THE serological technic has been used in taxonomic studies for more than fifty years. The discovery of precipitins by Krause in 1897 and the publication in 1904 of Nuttall's book applying the new technic to problems of animal relationships gave taxonomists an approach which showed great promise of clarifying disputed or undetermined relationships. The precipitin technic has been used to a limited extent for the latter purpose, but has been quite extensively applied for verification of existing relationships based on morphological criteria.

The usefulness and trustworthiness of the technic have been shown by many workers. Studies have chiefly been made with vertebrates but there has also been research with a few of the invertebrate phyla. The following is a brief list of some representative papers in the field: Boyden (1926, 1934, 1943), Boyden & Noble (1933), Wolfe (1936), Brown & Heffron (1928), Eisenbrandt (1938), Wilhelmi (1940), Martin & Cotner (1934), Baier & Wolfe (1942), Gemeroy (1943) and Leone & Pryor (1954). It seems to us that the greatest value of the serological technic now lies in its possibility of settling questionable animal or plant relationships.

Perhaps no other order of the Class Mammalia contains such a diversified group of animals as does the Order Carnivora. This diversity has been responsible for considerable conflict and uncertainty in attempts at classification. The problem lies not so much in the over-all picture of Carnivora classification as in the grouping of related forms in the suborders and superfamilies. Most authors seem to agree that the order should be divided into two suborders: the Fissipedia and the Pinnipedia. There are disagreements as to the relationships and inter-relationships among the families of the Fissipedia and the relationships of these families to the Pinnipedia. Winge (1923-24) and Scott (1937) both seem to favor combining the Ursidae, Canidae and Procyonidae into one group, with the Mustelidae as a distinct but associated family, and the Viverridae and Hyaenidae into another group, with the Felidae as a distinct but associated family. Winge associates the Pinnipedia with the Ursidae and Canidae. Beddard (1902), Romer (1933) and Simpson (1945) favor combining the Canidae, Ursidae, Procyonidae and Mustelidae into one superfamily and the Viverridae, Hyaenidae and Felidae into another superfamily. Beddard apparently believes the Pinnipedia closely associated with the Mustelidae, whereas Romer seems to indicate that the Pinnipedia are most closely associated with the Ursidae and Canidae. Other authors have opinions varying slightly from the above.

Since their discovery in the late Nineteenth Century, little use has been made of serological reactions in the taxonomic study of the Carnivora. Nuttall (1904) summarized the results of his flocculation tests with the sera of 56 different species of Carnivora. In general his results agreed with the systematic position of the species tested. With this lone exception no other extensive work has been done on the serological taxonomy of the Carnivora. Brief mention of them is made in several papers: Boyden (1926, 1942), Boyden & Gemeroy (1950) and Wolfe (1936).

An extraordinary study on the taxonomy of the Carnivora was included in the book by Reichert & Brown (1909) in which the relationships between various members were deter-

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mined by the resemblances and differences of hemoglobin crystals formed from the different species. On the basis of their study Reichert & Brown claimed that the Ursidae and Mustelidae showed a closer relationship to the Pinnipedia than did any of the other Fissipedia. A peculiar finding was that the hemoglobin crystals of the skunk more nearly resembled those of the Procyonidae, which in turn did not resemble those of the Pinnipedia. The hemoglobin crystals of the Canidae resembled those of the Pinnipedia less, while the crystals of the Felidae and Viverridae resembled those of the Pinnipedia least of all.

The classification and nomenclature used in this paper follow those of Simpson (1945) as much as possible.

Materials and Procedures

Many of the blood sera used as antigens were received from the New York Zoological Park through the courtesy of Dr. L. J. Goss and from the San Diego Zoo and the Serological Museum of Rutgers University through the courtesy of Drs. C. R. Schroeder and Alan A. Boyden respectively. Table 1 presents a list of animals from which sera were obtained.

Both chickens and rabbits were used for the production of antisera. Three different technics of precipitin testing were employed. These were the ring (interfacial) test, the photronreflectometer method of Libby (1938) and the microdensitometer method of Baier (1943). The latter two are turbidimetric methods; the photronreflectometer measures scattered light produced by the flocculating particles and the microdensitometer measures transmitted light. The authors found it advisable to use different injection procedures in order to obtain antisera of different precipitating ability, for it was necessary to have quite heavy precipitates when the microdensitometer was used, and weaker precipitating sera when the photronreflectometer was employed.

With one exception, all antisera used in the ring tests were produced in chickens. Each chicken was given a single intravenous inoculation of 1 ml. of a 2 per cent. solution of blood serum (the antigen). This injection procedure is the best for production of antiserum of low precipitating power, a high interfacial titer and good specificity (Wolfe, 1936). The birds were bled eight to ten days after the injection. The antisera were allowed to stand for at least seven days in the refrigerator before use, as *in vitro* changes occurred in the serum upon such standing (Wolfe, 1942). The one rabbit used was treated in the same manner

Table 1. Animals Used in Study

Order CARNIVORA
- Suborder Fissipedia
  - Family Canidae
    - *Canis familiaris* (dog)
    - *Canis lupus* (timber wolf)
    - *Vulpes fulva* (red fox)
  - Family Ursidae
    - **Ursus americanus* (black bear)
    - **Thalarctos maritimus* (polar bear)
  - Family Procyonidae
    - *Procyon lotor* (raccoon)
    - ***Potos caudivolvulus* (kinkajou)
    - ***Nasua narica* (coati-mundi)
  - Family Mustelidae
    - *Mustela furo* (ferret)
    - *Mephitis mephitis* (skunk)
    - *Taxidea taxus* (badger)
    - *Mustela vison* (mink)
    - **Tayra* sp. (tayra)
  - Family Felidae
    - **Felis concolor* (mountain lion)
    - *Felis domesticus* (house cat)
    - *Panthera pardus* (leopard)
    - **Panthera tigris* (tiger)
    - **Acinonyx jubatus* (cheetah)
  - Family Hyaenidae
    - ***Hyaena hyaena* (striped hyaena)
- Suborder Pinnipedia
  - Family Otariidae
    - ****Eumetopias jubata* (Steller's sea lion)
    - ****Zalophus californianus* (California sea lion)
    - **Zalophus californianus* (California sea lion)
  - Family Odobenidae
    - **Odobenus rosmarus* (walrus)
  - Family Phocidae
    - ****Phoca vitulina richardii* (harbor seal)

Order ARTIODACTYLA
- Family Bovidae
  - *Bos taurus* (cattle)
  - *Bison bison* (American bison)

Order PRIMATES
- Family Hominidae
  - *Homo sapiens* (man)

*Sera furnished by Dr. L. J. Goss.
**Sera furnished by Dr. A. A. Boyden.
***Sera furnished by Dr. C. R. Schroeder.
All other sera collected from local sources, including Madison Zoo.

described for production of antisera used in the photronreflectometer method.

Both chickens and rabbits were used in the production of antisera for the photronreflectometer studies. The rabbits were given a single series of three intravenous injections on alternating days, totaling 3 ml. of undiluted serum; the first injection was 0.5 ml., the second 1 ml. and the third 1.5 ml. The chickens received the same number of injections and on similar days but the solution was a 2 per cent. solution of the antigen rather than undiluted serum. The rabbits and chickens were bled on the seventh day after the last injection.

The antisera used in the microdensitometer studies were produced in chickens and rabbits. The increased precipitating power of the antisera needed in such studies was produced by increasing the amount of antigen inoculated into the animals. The rabbits were given two more series of three injections each at an interval of approximately 30 days. Each series consisted of a total of 3 ml. of undiluted serum. The chickens were given only one injection series, totaling 3 cc. of undiluted serum in three injections. The rabbits and chickens were bled seven days after the last injection. It should be emphasized that the above injection procedures did not always result in antisera of sufficient potency, and rather than reinject the animals that were poor antibody producers they were discarded. Chickens were found to be much better producers of antibody than the rabbits.

The chickens and rabbits were starved for 18 to 24 hours before bleeding. The blood was removed by cardiac puncture and allowed to clot. The serum was removed after centrifugation and stored in the refrigerator.

The ring test was performed in a 1.8 per cent. saline solution when chicken antiserum was used and in a .9 per cent. buffered saline solution when rabbit antiserum was employed. Serial dilutions of the antigen were made from a 2 per cent. solution which in turn was made from the undiluted antigen. One-tenth of a milliliter of antisera was layered below the antigen solutions (.5 ml.). Readings were made at 5, 10, 20, 30 and 60 minutes, but only the 60-minute readings are recorded in this paper.

The test antigens employed in the photronreflectometer and microdensitometer studies were also serially prepared. The final reaction mixtures of antigen and antisera were approximately .9 per cent. for the rabbit system and 8 per cent. for the chicken system, since Goodman, Wolfe & Norton (1951) showed that 8 per cent. was the optimum for the chicken antiserum system. In order to conserve antiserum, only alternate dilution tubes were used in the microdensitometer and photronreflectometer tests.

The photronreflectometer tests were conducted according to a procedure modified slightly from that outlined by Baier (1947). The cells used were standardized as to thickness. This means that the light beam passes through the same distance in the liquid of each set of cells. This is important in measuring the light-scattering effect of particles in a suspension. Comparative results cannot be obtained if this distance varies among the individual cells of each set. The microdensitometer tests were conducted according to the procedure outlined by Baier (1947). The tubes used in these tests were standardized according to diameter and transmission of light beams.

The photronreflectometer and microdensitometer tests were made with antigen dilutions so chosen that the final readings for the highest and lowest dilutions (antibody excess and antigen excess) equalled the control reading at those two points. This was not always possible at the antigen excess region because of the small amount of antigen that was available in several cases.

Values of the relationships are expressed in percentage. The homologous reaction is considered to be 100 per cent. and the heterologous reactions are related to this. The galvanometer readings were summated for all the antigen concentrations used when the photronreflectometer and microdensitometer technics were used.

Results

Table 2 presents a summary of the results obtained with 15 different antisera using the ring test technic. The two anti-Mustelidae sera (anti-mink and anti-ferret) gave high cross-reactions not only with other mustelids but also with the dog, fox, black bear and raccoon. The degree of cross-reactions with members of the Felidae were considerably lower in two of the three tests made and the reactions with Bovidae and Hominidae were very low.

Only one Canidae antiserum was produced. The anti-red fox serum reactions indicated that the Ursidae and Procyonidae were more closely related to the Canidae than to the Mustelidae or Felidae.

The anti-black bear serum was a very specific serum and gave a high cross-reaction only with the raccoon serum. Much weaker reactions occurred with other families of Fissipedia and also with man.

TABLE 2. SEROLOGICAL RELATIONSHIPS AMONG THE CARNIVORA—RING TEST TECHNIC. RELATIONSHIP VALUES EXPRESSED IN PER CENT.

Antisera / Antigen Source	Mink-67 (12800)*	Ferret-107 (51200)	Fox-165 (25600)	Black bear-162 (12800)	Cat-65 (51200)	Leopard-139 (25600)	Mountain lion-111 (25600)	Kinkajou-PC-10 (51200)	Coati-mundi-PC-10 (51200)	Raccoon-C-1416 (51200)	Steller's sea lion-C-155 (51200)	Steller's sea lion-C-163 (51200)	Walrus-PC-88 (6400)	Harbor seal-C-165 (25600)	Harbor seal** 12800
Suborder FISSIPEDIA															
Family Procyonidae															
Kinkajou								100			6.3				6.3
Coati-mundi									100						6.3
Raccoon	50	100	25	25		6.3	2.3	50	50	100	12.5	0	25	0	0
Family Ursidae															
Polar bear								12.5		50	6.3	12.5	25	0.4	
Black bear		100	25	100		3.1		6.3	100	50		6.3	25	0.8	
Family Canidae															
Red fox	50	25	100	1.6		0.4		12.5		25		0	25	0	
Dog	25	50	50	3.1	6.3	1.6	0.8								0
Family Mustelidae															
Ferret	100	100	12.5		4.7	1.6				25					
Skunk	50	50	3.1	3.1	12.5	3.1	0.4	6.3	12.5	50	12.5	1.6	12.5	0	
Mink	100	100		3.1		1.6	1.6								
Badger	50	50	3.1	3.1	3.1	3.1	1.6								
Family Felidae															
Cat	9.4	18.8	3.1	1.6	100	12.5	50			6.3					0
Leopard			3.1	3.1		100									
Mountain lion		25		6.3	100	50	100								
Suborder PINNIPEDIA															
Family Otariidae															
Steller's sea lion								50	100	25	100	100	50	3.1	25
Family Odobenidae															
Walrus								25	50	25	50		100	3.1	25
Family Phocidae															
Harbor seal								25	50			25	50	100	100
Order ARTIODACTYLA															
Cattle	6.3				3.1	0	1.2	0.8	3.1			0.8	0	0	
Bison	6.3	0	1.6		6.3	0									
Order PRIMATES															
Man	6.3	0.9	0.8	3.1	3.1		2.3								

* Ring test titer.
** Produced in rabbit; all others produced in chickens.

Three anti-Felidae sera were tested. All of these gave strong cross-reactions with the cat, leopard and mountain lion, but a low order of reactions with the members of other families. The intra-family results were very peculiar in one test. The reactions of the anti-leopard serum indicated that the leopard was more closely related to the mountain lion than to the house cat. Such unexpected results warrant further investigation.

The three Procyonidae sera were all quite aspecific in their cross-reactions. All these sera gave large reactions with the Pinnipedia and the anti-kinkajou serum indicated that these Pinnipedia were more closely related to the kinkajou than were the Mustelidae, Canidae and Ursidae. The one test made with a representative of the Felidae showed the cat to be more distantly related to the raccoon than to other Fissipedia.

TABLE 3. SEROLOGICAL RELATIONSHIPS AMONG THE CARNIVORA—MICRODENSITOMETER AND PHOTRONREFLECTOMETER TECHNICS. RELATIONSHIPS EXPRESSED IN PER CENT.

	Microdensitometer					Photronreflectometer			
Antigen Source / Antisera	*Steller's sea lion-157	*Steller's sea lion-CU	Steller's sea lion-3698-3796	*Black bear-3659-3660	*Raccoon-CX	Steller's sea lion-5	*Wolf-PE	*Skunk-PB	*Cat-PA
Suborder FISSIPEDIA									
Family Procyonidae									
Kinkajou		42			65.7		16.7		1.37
Raccoon	11.6	23.4	49.2	34.9	100	18.2		0	0.7
Family Ursidae									
Polar bear	34.9	39	55.6	93	53.6	15.7	21.6	15.9	0
Black bear	26.7	35.9	59.5	100	26.9	18.4	18.8	11.2	22
Family Canidae									
Red fox	20.5	13.4	47.3	38.4	18.6		52.5		0
Dog						2.9	88.1		0
Timber wolf							100	5.	1.3
Family Mustelidae									
Skunk		30.7	55.3	45.3	37.3	20.1		100	2
Ferret						13.1	12.1	11.3	1
Tayra								15.1	
Family Felidae									
Cat						6.1	1	3.2	100
Tiger						0			50.4
Mountain lion									47.8
Cheetah									76.6
Family Hyaenidae									
Hyaena								0	18.3
Suborder PINNIPEDIA									
Family Otariidae									
Steller's sea lion	100	100	100	45.3	32.7	100	9.3	6	0
Cal. sea lion						82.6			.9
Family Odobenidae									
Walrus						45.5	8.9	8.1	
Family Phocidae									
Harbor seal	81.5	52.4	76.6	44.2		47.4	9	10.3	
Order ARTIODACTYLA									
Cattle		14.4	17.7	5.2	4.5				

* Antisera produced in chickens; other produced in rabbits.

Five anti-Pinnipedia sera were tested. They all gave distinct suborder reactions. One of the two anti-sea lion sera was more specific than the other and its reactions indicated that the Ursidae were more closely related to the sea lion than was the raccoon, fox or skunk. The anti-walrus sera was aspecific; it gave fairly distinct subordinal reactions but the degree of reactions with representatives of the Fissipedia showed a similarity of the relationships of all four families of this suborder to the Pinnipedia.

The reactions of the two harbor seal antisera were interesting. The antiserum (C-165) produced in the chicken was highly specific and its

reactions with the blood of the sea lion and walrus were very low. It gave no reactions with the raccoon, fox or cat and only slight reactions with the polar bear and black bear. On the other hand, the antiserum produced in the rabbit gave reactions with the kinkajou and coati-mundi but not with the raccoon, dog or cat. No explanation suggests itself for the differences shown by these two antisera.

Table 3 records the results secured with the turbidimetric technic. Nine antisera were used and of these four were produced against the sea lion. These four antisera gave definite subordinal reactions and the three that were tested by the microdensitometer method indicated that the Ursidae were probably more closely related to the Pinnipedia than were the red fox or raccoon. On the other hand reactions with antiserum-CU were slightly higher for the kinkajou than with the Ursidae and antiserum 3698-3796 gave reactions with the skunk sera that were of similar magnitude to that of the Ursidae.

The black bear antiserum gave distinct familial reactions and the cross-reactions with the Pinnipedia were slightly higher than with the raccoon, red fox and skunk. The high degree of cross-reaction with the polar bear could indicate that a closer relationship exists than the present classification of these animals indicates. It would be unsafe to argue this question on the evidence of but one antiserum.

The anti-raccoon serum results showed the expected close relationship between the raccoon and the kinkajou. The Ursidae, Mustelidae and Pinnipedia showed a closer relationship to the Procyonidae than did the Canidae. In this particular series of tests the great difference between the polar bear result and the black bear result is strange considering the close relationship shown between these two animals in other tests. The authors cannot account for this variation but future tests may give a satisfactory answer.

The anti-wolf serum results gave an excellent example of intra-family relationships. The dog showed an 88 per cent. relationship to the wolf, while the red fox showed only a 52 per cent. relationship. Of the families tested, the Ursidae and Procyonidae seemed to be more closely related to the Canidae than were the mustelids. The representatives of three families of Pinnipedia showed a remarkably similar relationship to the wolf, while the cat (Felidae) was most distantly related.

The anti-skunk serum had relatively low precipitating power and was highly specific. The ferret and the tayra are members of the subfamily Mustelinae while the skunk belongs to the subfamily Mephitinae. The ferret and tayra showed no closer relationship to the skunk than did the Ursidae. The Pinnipedia, Canidae and Felidae showed distant relationship while the raccoon and hyaena gave no cross-reactions.

The anti-cat serum results showed strong intra-family relationships but extremely weak inter-family relationships with all but the Hyaenidae. The intra-family results were peculiar. The cheetah showed a 76 per cent. relationship to the house cat while the tiger showed a 50 per cent. relationship and the mountain lion showed a 47 per cent. relationship. From these limited data one gets the impression that the tiger and mountain lion might belong to a different genus than the house cat. These results warrant much more research.

Discussion

The findings set forth above are at least a beginning in the serological study of the relationship of the Carnivora. The species used in the study were chosen primarily because of their availability.

As indicated previously, authorities disagree about the relationship of the families of the Fissipedia to each other and to the Pinnipedia on the basis of comparative anatomy and paleontology. This has resulted in great variations and confusion in the classification and nomenclature of the Order Carnivora. On the basis of the serological data presented in this study, the following tentative conclusions can be drawn:

1. The Pinnipedia are more closely related to the Ursidae, Canidae, Mustelidae and Procyonidae than to the Felidae and Hyaenidae.

2. The closest serological resemblance exists between the Ursidae and Pinnipedia.

3. The Felidae and Hyaenidae are more closely related to each other than to any other family.

4. The polar bear seems to be quite closely related to the black bear and possibly should be included in the same genus.

If additional data support present findings, it might mean that the nomenclature of members of the family Felidae should be revised. Simpson in his work on the classification of mammals came to this same conclusion in his discussion of the taxonomy of the Felidae.

To attempt a serological classification of the Carnivora on the basis of the results presented in this paper might be premature. However, certain indications are given by these somewhat limited data. The tendency to classify the Canidae, Ursidae, Procyonidae and Mustelidae together into one superfamily (Canoidea) is perfectly valid serologically. Whether the Felidae,

Hyaenidae and Viverridae can be classified together into another superfamily serologically will have to be determined in the future. There are very strong indications that the suborders Fissipedia and Pinnipedia do not exist serologically. Future research may show that there are three or even four serological suborders of the Carnivora.

The authors realize the shortcomings of this paper as well as other serological research in which animal relationships have been considered. The shortcomings are due to the lack of materials to make more complete studies. It would be much more preferable to concentrate on fewer species but with larger numbers of specimens so that a number of antisera could be produced against each species and tested against several members of each studied. In this way it might be possible to make a statistical analysis of the data and one that might yield significant results rather than mere indications.

Summary

1. Serological tests were carried out using the blood sera of 23 different species from nine families of the Order Carnivora, two sera of the Order Artiodactyla, and human serum (Order Primates).

2. The ring test, the Baier microdensitometer and the Libby photronreflectometer were all used in performing these tests.

3. Where comparable reactions were made, the results of the microdensitometer and photronreflectometer tests paralleled those of the ring test.

4. A serological basis for the classification of the Carnivora was indicated.

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